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## *Hansenula polymorpha*: An attractive model organism for molecular studies of peroxisome biogenesis and function

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### 1. SUMMARY

In wild-type *Hansenula polymorpha* the proliferation of peroxisomes is induced by various unconventional carbon- and nitrogen sources. Highest induction levels, up to 80% of the cytoplasmic volume, are observed in cells grown in methanol-limited chemostat cultures. Based on our accumulated experience, we are now able to precisely adjust both the level of peroxisome induction as well as their protein composition by specific adaptations in growth conditions.

During the last few years a series of peroxisome-deficient (*per*) mutants of *H. polymorpha* have been isolated and characterized. Phenotypically these mutants are characterized by the fact that they are not able to grow on methanol. Three mutant phenotypes were defined on the basis of morphological criteria, namely: (a) mutants completely lacking peroxisomes (*Per*<sup>−</sup>; 13 complementation groups); (b) mutants containing

few small peroxisomes which are partly impaired in the peroxisomal import of matrix proteins (*Pim*<sup>−</sup>; five complementation groups); and (c) mutants with aberrations in the peroxisomal substructure (*Pss*<sup>−</sup>; two complementation groups). In addition, several conditional *Per*<sup>−</sup>, *Pim*<sup>−</sup> and *Pss*<sup>−</sup> mutants have been obtained. In all cases the mutant phenotype was shown to be caused by a recessive mutation in one gene. However, we observed that different mutations in one gene may cause different morphological mutant phenotypes. A detailed genetic analysis revealed that several *PER* genes, essential for peroxisome biogenesis, are tightly linked and organized in a hierarchical fashion.

The use of both constitutal and conditional *per* mutants in current and future studies of the molecular mechanisms controlling peroxisome biogenesis and function is discussed.

### 2. INTRODUCTION

Microbodies (peroxisomes) are ubiquitous single-membrane bound organelles, present in all eukaryotes examined so far except archaezoa [1].

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Despite their simple morphology, the organelles show a great diversity in enzyme repertoires and, as a consequence, may be involved in a variety of metabolic processes [2–4]. These functions are partly inducible in nature and may also vary with the developmental stage and/or growth conditions of the related organism. This multi-functionality of microbodies is unique among subcellular organelles in eukaryotes. Their importance in cellular metabolism may be stressed by the fact that peroxisomal dysfunction often leads to metabolic abnormalities and may be lethal, particularly in man (Zellweger syndrome; [5]).

In yeasts, microbodies were first described by Avers [6]. In the intervening years much progress has been made towards our understanding of the mechanisms involved in biogenesis and metabolic function of these organelles [4,7–10].

The aim of this contribution is to summarize major recent developments in the studies on peroxisome biogenesis/function in the methylotrophic yeast *Hansenula polymorpha* and to emphasize the potential of this organism as a

model system in future studies on this comparatively neglected organelle.

### 3. PEROXISOMES IN WILD-TYPE CELLS OF *H. POLYMORPHA*

#### 3.1. Peroxisome induction and morphology

In yeasts the proliferation and metabolic significance of peroxisomes is largely prescribed by cultivation conditions [7–10]. In *H. polymorpha* optimal induction (up to 80% of the cytoplasmic volume) is achieved during growth of cells on methanol [7]. Other substrates known to induce microbodies in this organism are ethanol, primary amines, D-amino acids and purines [7,10] but not oleic acid [11]. Peroxisomes play a crucial role during growth of cells under these conditions since they contain key enzymes of the metabolism of the above carbon or nitrogen sources. The current view on the compartmentalization of methanol metabolism is depicted in Fig. 1.

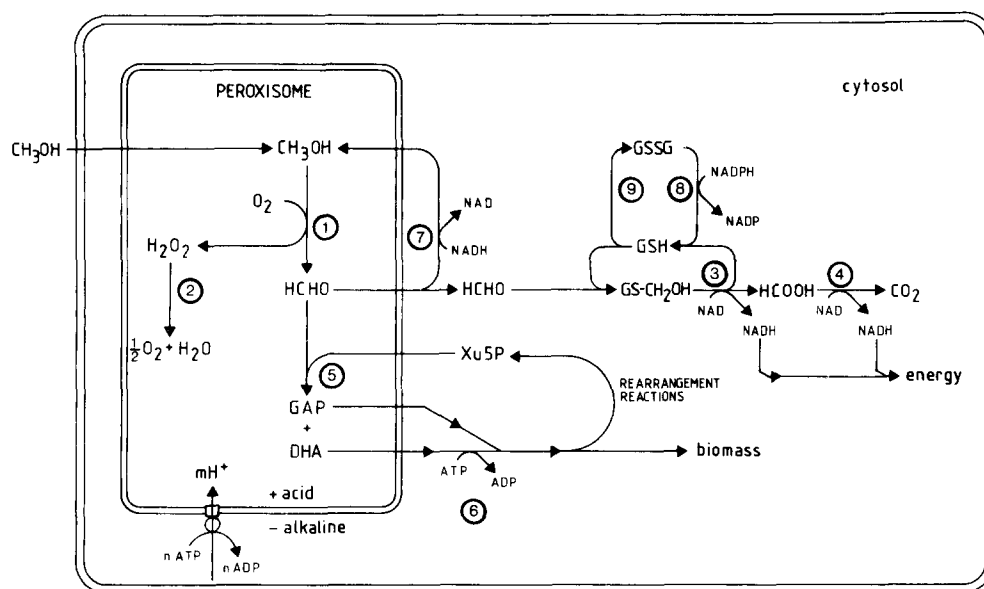


Fig. 1. Schematic representation of the compartmentalization and the function of peroxisomes in methanol metabolism in *H. polymorpha* WT cells. 1. alcohol oxidase; 2. catalase; 3. formaldehyde dehydrogenase; 4. formate dehydrogenase; 5. dihydroxyacetone synthase; 6. dihydroxyacetone kinase; 7. formaldehyde reductase; 8. glutathione reductase; 9. oxidation of glutathione (reproduced from ref. 41).

The mature size and substructure of peroxisomes contained in *H. polymorpha* is also a reflection of the cultivation conditions and is particularly determined by: (i) the nature of the carbon/nitrogen source used for growth [7,10]; and (ii) the mode of cultivation and the growth phase of the culture [10]. Large cuboid, completely crystalline organelles (Fig. 2) were only observed in cells grown in methanol-limited chemostat cultures at low dilution rates and are due to the synthesis (and crystallization) of excessive amounts of alcohol oxidase protein. In cells from the exponential phase in batch cultures, rounded organelles are predominant; this morphology is largely independent of the growth substrate. Differences in shape between individual organelles present in one cell may furthermore reflect differences in their developmental stage [12].

### 3.2. Development of peroxisomes

There is now a general consensus that peroxisomes in wild-type (WT) yeast cells develop from mature, pre-existing organelles [4,7,8]. In *H. polymorpha*, peroxisome proliferation has been studied by ultrastructural methods following a shift of cells from glucose to methanol-containing media.

In glucose-grown cells (Fig. 2A) generally a single, small peroxisome is present in each cell [10]. Upon a shift of cells to methanol this organelle rapidly increased in size due to the import of newly synthesized matrix proteins (e.g. alcohol oxidase and dihydroxyacetone synthase). Growth of the organelles continued until a mature peroxisome was formed from which small peroxisomes were separated, which in turn grew. The results obtained indicated that the capacity to import proteins was strictly associated with the capacity of the organelle to divide. As a consequence, the microbody population in one cell is heterogeneous [12]; only small immature organelles are capable of incorporating newly synthesized proteins and of subsequent fission. This mechanism of microbody multiplication is probably general in yeasts [13].

Proliferation of peroxisomes is not simply triggered by the synthesis of their matrix proteins; in different species (over)expression of matrix proteins only resulted in a considerable increase in size but not in the number of organelles; this implies that, upon saturation of the import capacity of the organelles, additionally expressed matrix proteins will remain in the cytosol where they

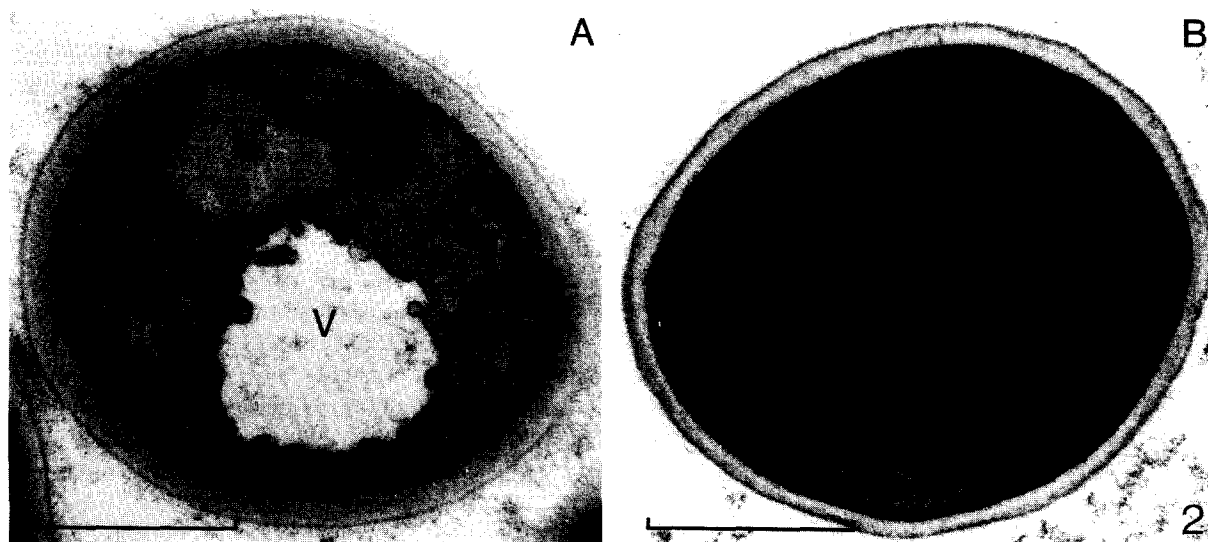


Fig. 2. Survey of cells of *H. polymorpha* from a batch culture in the mid-exponential growth phase on glucose (A) and from a methanol-limited chemostat culture (B) to show the overall cell morphology and the shape of peroxisomes. N, nucleus; P, peroxisome; V, vacuole. Bar = 1  $\mu$ m.

generally accumulate into large proteinaceous aggregates [14,15]. Furthermore, fission of peroxisomes is also not dependent on the size of the organelles as was evident in mutants of *H. polymorpha* which are partly blocked in matrix protein import [16].

### 3.3. Peroxisomal protein targeting and assembly

The activity of peroxisomal matrix enzymes in WT cells is strictly confined to the peroxisomal matrix [7]. Peroxisomes lack nucleic acids and ribosomes; therefore all peroxisomal proteins are encoded by nuclear genes. Precursors of these proteins are synthesized in the cytosol on free polysomes and imported post-translationally into the target organelle, where assembly and activation takes place. Precursors of matrix proteins do not contain cleavable targeting sequences; consequently, topogenic information has to be contained in the amino acid sequence of these proteins. Recently, a highly conserved, general targeting signal (PTS) was identified, located at the extreme C-terminus of various matrix proteins (SKL-COOH; [17,18]). However, this SKL-motif is certainly not the only peroxisomal targeting signal. This is indicated by the finding that not all peroxisomal proteins contain an SKL or SKL-like motif which is particularly true for yeast enzymes [19,20]. Indeed, other sorting signals have been identified [21]. These signals also are predominantly located at the extreme C-terminus although exceptions have been encountered [22]. In baker's yeast the latter may be true for thiolase [9], whereas in *H. polymorpha* amine oxidase does not contain a C-terminal PTS (Faber et al., submitted).

Additional components essential for protein import/assembly are probably constitutively present in *H. polymorpha* since both alcohol oxidase and dihydroxyacetone synthase, artificially expressed under non-methylotrophic growth conditions, were nevertheless imported and correctly assembled into the active protein inside the target organelle [24,25]. On the other hand, amine oxidase, when artificially expressed under ammonium excess conditions (conditions under which the homologous gene is fully repressed), was only partially imported in *H. polymorpha*. However,

this part-inhibition of amine oxidase import was fully abolished in the presence of an amine substrate (K.N. Faber and M. Veenhuis, unpublished results). The above phenomena might be explained by the presence of different receptor/translocator proteins for distinct classes of precursors (e.g. based on similarities in targeting sequences). This is not a hypothetical possibility as examples exist, for instance, in mitochondrial protein import (recently reviewed by Wienhues and Neupert [26]). So far, two receptor proteins have been identified on the outer membrane of mitochondria of *Neurospora crassa*. MOM 19 serves as 'master receptor' and functions in the specific recognition of most mitochondrial precursors, including those that carry an N-terminal targeting sequence. Another receptor protein, MOM72, specifically recognizes the inner membrane-bound ATP-ADP carrier (AAC), which contains an internal targeting signal. However, MOM72 mutants still display a residual AAC import which now occurs via MOM19. Evidently, MOM19 can substitute for MOM72, although at low efficiency. By analogy, import of AO and DHAS in *H. polymorpha*, which both contain a C-terminal PTS [21], may be facilitated by a general, constitutive import mechanism, suggested before [16] which may partly substitute for a specific, substrate inducible, receptor for amine oxidase. A most intriguing question, however, remains whether different receptors interact with a common protein translocation machinery. Also, whether protein import/assembly is dependent on the energy status of the target organelle is still a matter of debate [27-29].

### 3.4. Degradation of peroxisomes

As a rule, peroxisomal enzymes are not inactivated after a shift of cells to a new growth medium in which these enzymes have become redundant. Generally, the observed decrease in their specific activities can be accounted for by dilution of existing enzyme protein over newly formed cells following repression of their synthesis in the new environment. However, two main exceptions have been encountered. These include selective inactivation (e.g. by excess glucose) of: (i) alcohol oxidase in *H. polymorpha*; and (ii) amine oxidase in

*Trichosporon cutaneum* [10]. In both organisms a rapid degradative turnover of the peroxisomal population was observed under these conditions. This process was energy-dependent but independent of protein synthesis. Peroxisomes were degraded individually by means of an autophagic process; hydrolytic enzymes required for the

degradation of the peroxisomal contents were supplied by the vacuole [30].

The mechanisms triggering peroxisome turnover are still unknown. However, cytosolic peroxisomal enzymes in peroxisome-deficient mutants of *H. polymorpha* (both *Per*<sup>-</sup> and *Pim*<sup>-</sup> mutants; for details see below) are no longer susceptible to

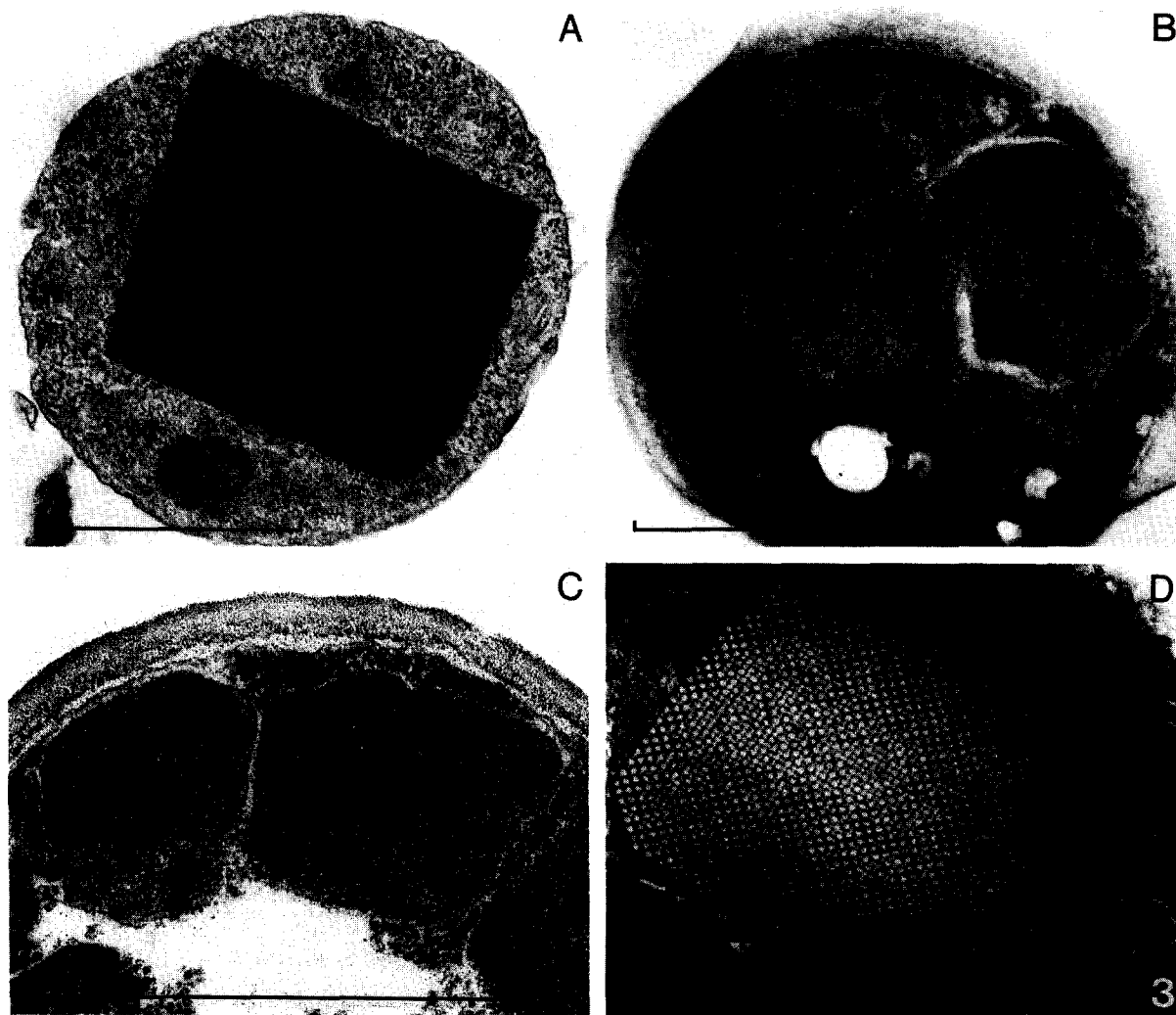


Fig. 3. (A) Typical morphology of a *Per*<sup>-</sup> mutant of *H. polymorpha* which completely lacks peroxisomal structures; when grown in chemostat cultures on glucose/methanol mixtures these cells contain large crystalloids, located in both the cytosol and the nucleus (A) spheroplast; glutaraldehyde/ $\text{OsO}_4$ ), which are composed of alcohol oxidase protein (D) ultrathin cryosection; uranyl acetate). *Pim*<sup>-</sup> mutants contain small peroxisomes ((B) arrows) which contain alcohol oxidase while bulk of the enzyme is located in the cytosolic and nucleus-bound crystalloids (protein A/gold; anti-alcohol oxidase). (C) Peroxisomal morphology in a *Pss*<sup>-</sup> mutant of *H. polymorpha* which is characterized by electron dense aggregates in the crystalline matrix (spheroplast, glutaraldehyde/ $\text{OsO}_4$ ). A, cytosolic alcohol oxidase crystalloid; N, nucleus; V, vacuole. Bar = 1  $\mu\text{m}$  unless otherwise stated.

carbon catabolite inactivation [31]. These results indicate that the mechanisms initiating proteolytic turnover of peroxisomal enzymes are not directed against the enzyme protein, but instead to the peroxisomal membrane.

#### 4. PEROXISOME DEFICIENT MUTANTS OF *H. POLYMORPHA*

##### 4.1. Mutant isolation and phenotypical characterization

All mutants described were identified within a collection of 260 methanol-utilization-defective ( $Mut^-$ ) mutants of *H. polymorpha*, previously described [32]. After incubation of the mutants in methanol-containing media, 85 strains were identified by electron microscopy as having one of the following peroxisomal defects: (1) complete absence of peroxisomes ( $Per^-$  phenotype; 58 strains); (2) the presence of a few small peroxisomes in conjunction with the presence of the bulk of the peroxisomal matrix proteins in the cytosol ( $Pim^-$  phenotype; 20 strains); and (3) aberrations in the peroxisomal substructure, i.e. presence of electron-dense inclusions in the crystalline peroxisomal matrix ( $Pss^-$  phenotype; seven strains). In addition, a series of conditional mutants (six complementation groups) have been isolated. The mutant phenotypes were shown to be determined by single recessive nuclear mutations. The different mutant phenotypes are described in more detail below.

**4.1.1.  $Per^-$  phenotype.** The  $Per^-$  mutants were organized into 13 different complementation groups. Representatives of the different groups showed comparable morphological phenotypes in that: (i) their growth is impaired on methanol, but all grow well on a range of other compounds including those that require the activity of peroxisomal enzymes in WT cells; (ii) all peroxisomal matrix and membrane proteins tested are normally induced and assembled; and (iii) all peroxisomal proteins, both inducible and constitutive, are located in the cytosol. Peroxisomal membrane proteins were located in small proteinaceous/phospholipid aggregates (Sulter et al., submitted). In fully derepressed cells cytosolic alcohol oxi-

dase forms large crystalloids (Fig. 3A,D) in which bulk of the other matrix proteins, except catalase, is also incorporated [34].

**4.1.2.  $Pim^-$  phenotype.** The  $Pim^-$  mutants were organized into five different complementation groups which, as  $Per^-$  mutants, showed mutual comparable phenotypes. The growth properties and peroxisomal enzyme expression levels between  $Pim^-$  and  $Per^-$  mutants are fully comparable. The major difference between the two classes of mutants is that in  $Pim^-$  mutants a few small peroxisomes were still present. All peroxisomal proteins tested, both inducible and constitutive, were located in both these organelles and the cytosol (Fig. 3B), indicating a defect in a general major import mechanism.

**4.1.3.  $Pss^-$  phenotype.** These mutants show normal peroxisome proliferation on induction of cells on methanol but are characterized by abnormalities in organellar shape and matrix substructure, due to improper assembly of part of the matrix protein (Fig. 3C). Since all enzymes involved in methanol metabolism are present and active at their correct location, we speculate that dysfunctioning of the organelles is due to a defect in peroxisome transport properties (e.g. dissipation of the proton motive force across the peroxisomal membrane [27]).

##### 4.1.4. Conditional mutants.

- (i) Temperature-sensitive ( $Ts$ ) mutants: one group of  $Ts$  mutants show the  $Per^-$  phenotype (and fully lack peroxisomes) at the restrictive temperature ( $43^\circ C$ ) but have WT properties (and contain intact peroxisomes) at permissive growth conditions ( $< 37^\circ C$ ). Surprisingly, at intermediate temperatures (between  $37-43^\circ C$ ) steady state conditions have been obtained in which the cells display the  $Pim^-$  phenotype.
- (ii) pH-mutants: based on the screening procedure, developed for the isolation of vacuolar pH-mutants of *S. cerevisiae* [35], different peroxisomal pH-mutants of *H. polymorpha* were isolated. Mutants were obtained which were unable to grow on methanol at pH 7.0 but show the normal WT phenotype at pH 5.0. This pH-effect is specific for utilization of methanol: growth on other substrates which

require the activity of peroxisomal enzymes is not affected. In these mutants the peroxisomal proliferation and protein composition is unaltered: at both pH values the different key enzymes of methanol metabolism are peroxisome-bound. However, at pH 7.0 improperly assembled matrix protein (e.g. alcohol oxidase) was observed, whereas the enzyme was normally oligomerized in cells from cultures grown at pH 5.0. Therefore, these mutants appeared to be impaired in protein assembly rather than protein import.

#### 4.2. Genetic analysis

As stated above, all *per* mutant phenotypes are due to monogenetic defects; this implies that the absence (or incorrect synthesis) of a single gene product may cause the absence of a complete organelle (the peroxisome). This unique property renders these mutants very attractive for a molecular analysis of peroxisome biogenesis.

All *PER* genes have been subjected to a detailed genetic analysis. Complementation and linkage analysis showed that the total *per* mutant population represented mutations in 14 genes. Figure 4 shows the linkage relationships and map positions of the corresponding genes, as deduced from random spore analysis. Twelve genes were defined, designated *PER1* through *PER12*; ten of these genes were mapped in three separate linkage fragments (Fig. 5). Two genes, *PER8* and *PER12*, were unlinked to each other, to any of the *PER* genes indicated below (Fig. 5), or to *LEU1* and *ADE11* loci. In addition to defining the number and relative positions of *PER* genes, the combined complementation and genetic linkage analysis also made clear that *Pim*<sup>-</sup>, *Per*<sup>-</sup> and *Pss*<sup>-</sup> phenotypes were allele-specific and not gene-specific since, in at least two instances, different alleles of one gene displayed different peroxisome-defective phenotypes [36] indicating that the protein products encoded by these genes may represent multifunctional components which participate in different aspects of peroxisome assembly/proliferation. A comparable model of functional multiplicity has, for instance, been proposed to explain defects in both Golgi to vacuole

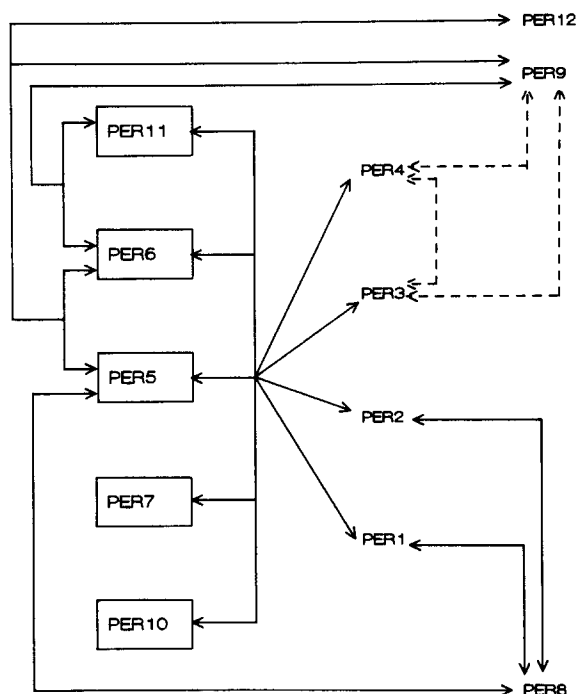


Fig. 4. Interactions between products of *PER1* to *PER12* genes deduced from unlinked non-complementation data. The dotted lines indicate 'weak' interactions ('leaky' growth of the corresponding double heterozygous hybrids at restrictive temperatures). From ref. 38.

sorting and vacuole segregation caused by *vac1* mutations [37].

These studies furthermore revealed many cases of an unusual complementation behaviour, namely unlinked non-complementation — defined as the failure of recessive mutations in different genes to complement — between different *per* alleles, especially at low temperature thus suggesting functional and physical links between their gene products (Titorenko et al., submitted). A map of interactions of products of these genes is depicted in Fig. 4.

### 5. FUNCTIONAL ASPECTS OF PEROXISOME METABOLISM

Physiological studies indicated that not all functions which are mediated by intact peroxisomes in WT cells are impaired in *Per*<sup>-</sup> mutants of *H. polymorpha*. For instance, all mutants grow



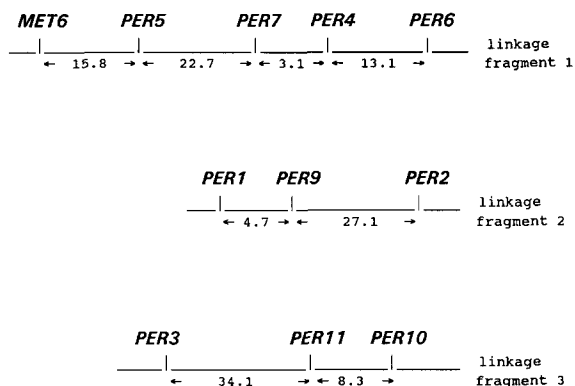


Fig. 5. Linkage relationships and map positions of *PER1*–*PER12*, as deduced from random spore analysis. (Titorenko et al., submitted). Gene order was determined by three-point analysis. Distance indicated in centi-Morgans *PER8* and *PER12* showed no linkage nor were they linked to any of the other *PER* genes or to *LEU1* and *ADE11* loci. Linkage fragment 1 was previously identified by Gleeson and Sudbury [46].

well on glucose in the presence of different organic nitrogen sources which require the activity of peroxisomal enzymes in WT cells (e.g. (m)ethylamine and D-amino acids) [39]. Therefore, different peroxisomal enzymes may also effectively

function in the cytosol. However, growth on other substrates is reduced (ethanol) or fully impaired (methanol) despite the fact that activities of all enzymes involved in the metabolism of these compounds are present in the cytosol. Therefore, these mutants are suitable model systems to study functional aspects of peroxisomes focussing on the fundamental question of the possible advantage for the organism to compartmentalize certain metabolic pathways. The results obtained so far indicate that these advantages—at least partly—may vary with growth conditions. In the case of ethanol metabolism the main advantage of intact microbodies is that it enables the cell to adjust the levels of different intermediates required to generate aspartate (the final product of microbody  $C_2$ -metabolism) from isocitrate and, in particular, to prevent a drain of oxaloacetate into other metabolic pathways [40]. Methanol however, cannot be used as carbon source by *per* mutants but may serve as an additional energy source in glucose-limited chemostat cultures [41]. These studies showed that intact, functional peroxisomes are indispensable to support growth of *H. polymorpha* on methanol (Fig. 6) and sug-

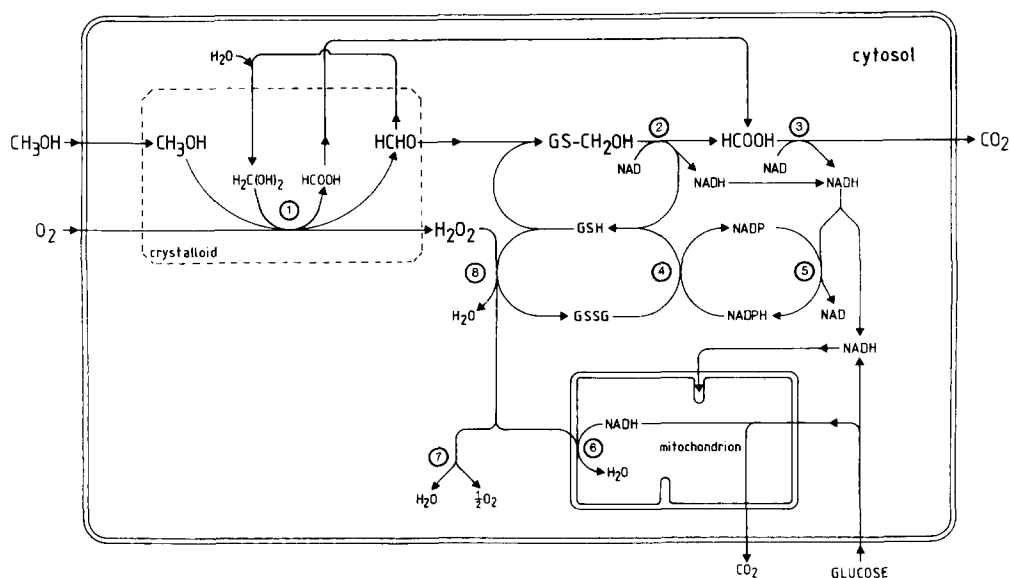


Fig. 6. Hypothetical scheme of methanol metabolism in a peroxisome-deficient mutant of *H. polymorpha*. 1. alcohol oxidase (catalyzes both the oxidation of methanol and methylene glycol, the hydrated form of formaldehyde); 2. formaldehyde dehydrogenase; 3. formate dehydrogenase; 4. glutathione reductase; 5. NADH/NADPH transhydrogenase; 6. cytochrome *c* peroxidase; 7. catalase; 8. chemical oxidation of glutathione by  $H_2O_2$  (reproduced from ref. 41).

gested that the essential functions of peroxisomes in methanol metabolism were twofold, namely to facilitate: (i) proper partitioning of formaldehyde, generated from methanol, over the dissimilatory and assimilatory pathways; and (ii) decomposition (by catalase) of  $H_2O_2$  at the site where it is produced, thus preventing decomposition of hydrogen peroxide by energy consuming processes.

## 6. PEROXISOME BIOGENESIS IN A PEROXISOME-DEFICIENT Ts MUTANT OF *H. POLYMORPHA*

As indicated, different Ts mutants of *H. polymorpha* are available which completely lack peroxisomes at restrictive temperatures (43°C) but show the WT phenotype at permissive growth conditions (< 37°C). Kinetic studies indicated that, after a shift of cells growing in methanol-containing media at 43°C to permissive growth conditions, new peroxisomes were rapidly formed. Development of these organelles appeared to be dependent on protein synthesis. The fate of mature matrix and membrane proteins, present in the cytosol prior to the shift of cells to permissive growth conditions was studied in detail in a constructed mutant which contained a heterologous peroxisomal membrane protein (the 47 kDa PMP from *Candida boidinii* [42]) as marker. Recent studies showed that PMP47 is correctly sorted to the peroxisomal membrane of WT cells and is located in protein/phospholipid aggregates together with homologous peroxisomal membrane proteins (PMPs) in *Per*<sup>-</sup> mutants of *H. polymorpha* (G. Sulter, unpublished results).

Transformants of Ts6, containing the 47 kDa PMP gene from *C. boidinii* under control of the *H. polymorpha* alcohol oxidase (MOX) promotor, were grown at restrictive temperatures under conditions that alcohol oxidase and PMP47 were expressed. Subsequently, samples of these cells were placed into a new growth environment composed in such a way that: (i) the MOX promotor is fully repressed (by excess glucose); and (ii) the synthesis of a new matrix enzyme (amine oxidase) is induced (by ethylamine) and incubated at permissive conditions in order to allow peroxisome

development. A detailed biochemical and ultrastructural analysis on the subcellular location of alcohol oxidase, amine oxidase and PMP47 in the initial hours of permissive growth in the new environment indicated that neither alcohol oxidase nor PMP47 were incorporated in the newly formed peroxisomes which contained amine oxidase and catalase activity; instead, both the already existing alcohol oxidase and PMP47 remained, virtually unaffected, in the cytosol (H. Waterham and M. Veenhuis, unpublished results). Taken together, these results suggest that de novo synthesis of peroxisomes in *H. polymorpha* might be possible.

## 7. CONCLUDING REMARKS

In the past few years much progress has been made towards the elucidation of various aspects of peroxisome biogenesis and function. Yeasts have developed into very useful model organisms for these studies because they combine a number of distinct advantages: ease of growth, accessibility to both classical and molecular genetics and the possibility to manipulate peroxisome proliferation and their protein composition. Moreover, now that numerous peroxisome-deficient mutants (*per* and *pas* mutants) are available of different yeasts, these organisms may become more and more important as a tool for cloning *PER/PAS* homologues of other sources by functional complementation of the corresponding yeast mutants with a heterologous genomic bank. Undoubtedly, future studies will not only continue to analyze the functional role of the various available *PER/PAS* genes in peroxisome proliferation/function but also focus on the mechanisms controlling protein import and assembly (role of cytosolic and peroxisomal chaperon(in)s, receptor/translocation mechanisms). A major obstacle for a rapid expansion of this field is the absence of reliable in vitro assays. Despite some recent progress, the efficiencies of these systems are very low, especially for yeast. Also, establishing unambiguous import criteria akin to, for instance, mitochondria (e.g. protein modification or pro-

cessing) and criteria for the intactness of peroxisomes are essential in this respect.

Until now, isolated yeast peroxisomes or peroxisomal membranes are invariably leaky [43] and all attempts to restore the original *in vivo* energization of the peroxisomal membrane so far failed [44]. However, recent studies indicated that the permeability of peroxisomes of *H. polymorpha* is attributed to the presence of a pore-forming protein. This protein, a constitutive 31 kDa integral peroxisomal membrane protein (PMP) has been purified and biochemically characterized [44]. The possibility to regulate this porin [44] has now opened the way for future studies on the possible energy dependency of various transport processes (protein import as well as transport of substrates and metabolic products/intermediates) across the peroxisomal membrane. The purpose of this contribution was to demonstrate that *H. polymorpha* is a highly attractive model organism for these studies. Not only has it the advantage that it harbours various crucial peroxisomal functions which can readily be manipulated, but also the availability of various mutants affected in peroxisome assembly and function ([16,32]; Titorenko et al., submitted), the identification of physical interactions between different *PER* gene products (Titorenko et al., submitted) in conjunction with recent major advances in the molecular genetics [45] and the extensive knowledge on the physiology/biochemistry of the organism [7,10] renders *H. polymorpha* an organism of choice for such studies.

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